Evidence of Mouse Epidermal Subpopulations with Different Cell Cycle Times

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In order to obtain information on the distribution of total cell cycle times in hairless mouse epidermis, basal cells were isolated and prepared for DNA flow cytometry at intervals after a pulse labeling with 50 μCi of thymidine. The DNA distributions were recorded, and cells were sorted from windows in the S, G_2, and G_1 phases of the cell cycle, collected on glass slides, and subjected to autoradiography. The proportions of labeled cells were scored in each fraction, and the percentage of labeled mitoses was determined in histologic sections from the same animals. Grain count distributions were recorded at selected time points over labeled cells in sorted fractions and over labeled mitoses. The movement of the labeled S-phase cohort was thus followed through all cell cycle phases. Peaks in labeled cells were observed at about 36 h in S phase, G_2 phase, and mitosis, and high levels of labeled G_2 cells and mitoses were seen at about 80 h. These results indicate the existence of one rapidly cycling subpopulation of keratinocytes with a cell cycle time slightly less than 30 h, in addition to keratinocytes with considerably longer cell cycle times. The first peak of labeled G_2 cells reached only about 30%. This is consistent with earlier findings of about 30% G_2 cells with a rapid traverse, and 70% with a considerably delayed traverse through G_2 phase. The proportion of labeled G_1 cells reached a value corresponding to twice the initial labeling index at 8 h after pulse labeling. This is consistent with previously obtained phase durations, indicating an unperturbed cell cycle traverse of labeled cells from S phase through G_2 and mitosis. *J Invest Dermatol 86:266–270, 1986*

Cells with long transit times in S and G_2 phase of the cell cycle were found in mouse epidermis [1,2]. Computer simulations indicated 40 and 70% of cells in S and G_2 phase, respectively, with considerably prolonged phase durations [3]. Whether subpopulations of epidermal cells exist with distinct differences in total cell cycle times, however, remains to be established.

To investigate this, the progression of a pulse-labeled S-phase cohort was followed through S, G_2, and G_1 phases of the cell cycle by DNA flow cytometry and cell sorting of isolated mouse epidermal basal cells. The cell cycle traverse through mitosis was followed by the percent labeled mitoses (PLM) method in histologic sections from the same animals. The percent labeled cells was determined by autoradiography in sorted fractions and among mitoses in histologic sections, respectively, at intervals up to 132 h after pulse labeling.

MATERIALS AND METHODS

Animals Hairless mice of both sexes of the *hr/hr* Oslo strain, 60–90 days of age, weighing about 25 g were used. They were kept 8 to a cage, had free access to food and water, and were exposed to a 12-h light/dark cycle with light from 0630 to 1830.

Cell Separation Slices of skin were cut from the backs of the animals with an electrokeratotome [4]. Basal cells were separated from differentiating cells by means of trypsin digestion [5] and shaken off into suspension [6]. The isolated basal cells were fixed by adding absolute ethanol dropwise while whirlmixing to a final concentration of 70–80%, and stored at 4°C as single cell suspensions.

DNA Flow Cytometry and Cell Sorting After fixation, basal cells were washed in saline, RNase-treated, and stained with ethidium bromide [6]. DNA frequency distributions were obtained and cells sorted in an Ortho Cytofluorograph 50 H (Ortho Instruments, Westwood, Massachusetts). Cells were sorted from windows comprising the left half of the G_1 peak, 15–20% of mid S, and the outer part of the G_2 peak and collected on glass slides for autoradiography (Fig 1). At least 10^3 cells were sorted from each fraction. To avoid sorting of doublets in the G_2 fraction, G_2 cells were discriminated from G_1 doublets by a 2-parameter plot of the pulse area against the pulse height. G_1 doublets thus appear as G_2 cells when the pulse area is measured alone, but are discriminated as G_1 cells because of the lower pulse height (Fig 2). Reanalysis of sorted G_2 populations showed an admixture of about 5% G_1 cells, indicating a high purity of the G_2 population [3].

Percent Labeled Mitoses A piece of back skin from each animal was fixed in Bouin's solution for 4 h, dehydrated, and embedded in paraffin. Histologic sections of 5-μm thickness were cut from each animal and processed for autoradiography.

[^{3}H]dThd Labeling and Autoradiography Animals were pulse labeled with 50 μCi [^{3}H]dThd (sp act 6.7 Ci/mmol; 1 mCi/ml, New England Nuclear Corporation, Boston, Massachusetts) in 0.3 ml 0.9% NaCl by i.p. injection at 0800. Groups of 6 animals were sacrificed by cervical dislocation at intervals up to 132 h after pulse labeling. Histologic sections and basal cells collected on glass slides were dipped in Kodak NTB 2 film emulsion diluted 1:1 with distilled water, exposed for 2 and 5 weeks, respectively, before being developed and stained with hematoxylin. Cells with

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Abbreviations:
MGC: mean grain count
PLM: percent labeled mitoses

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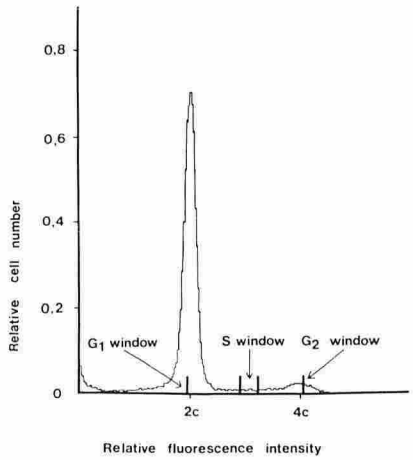

Figure 1. DNA distribution of isolated mouse epidermal basal cells indicating windows from which cells were sorted.

5 or more grains were considered as labeled. A negligible number of labeled cells had less than 4 grains in specimens collected shortly after the pulse. The percent labeled metaphases was scored among 30 metaphases in several sections from each animal. In sorted specimens the proportions of labeled cells among 300–500 cells were scored. In specimens from selected time points grain count distributions over labeled nuclei were constructed.

RESULTS

Labeling of Sorted Basal Cell Fractions Changes in the proportions of labeled cells with time after pulse labeling within the various cell cycle phases are shown for G_1 phase in Fig 3, and for S, G_2, and M phase in Fig 4.

The proportion of labeled cells in G_1 phase was 0.7% immediately after pulse labeling (Fig 3). This is the expected admixture of early S-phase cells into the G_1 fraction, which theoretically should not contain labeled cells 30 min after pulse labeling. The proportion of labeled cells reached about 15% at 12 h after pulse labeling, a level that was maintained until 120 h, whereafter an increase occurred.

The labeling index of nonsorted smears was 6.2% ± 0.8 SD. The proportion of labeled cells in mid-S 30 min after pulse labeling was about 80% (Fig 4, upper panel) and in accordance with previous results [2]. Then a drop occurred, followed by an increased level between 24–36 h and a subsequent slight decrease.

The proportion of labeled cells in G_2 phase (fig 4, center panel) reached a peak of only 30% 4 h after labeling, whereafter a reduction appeared. High levels were seen between 24 and 40 h followed by a trough and a subsequent third increase at about 72 h.

PLM Measurements (Fig 4, bottom panel) A first peak with about 85% labeled metaphases appears between 4–8 h, which is consistent with previous results [1]. Thereafter a second peak of 50% labeled cells is seen at 36 h, followed by a third and broader peak. The experiment was repeated from 24–44 h, with reproducible values with respect to all measured parameters. Hence the values from both experiments were pooled.

Animals with visible wounds or claw marks are not used in experiments. Microscopic evaluation of the epidermis with respect to hyperplasia, that might be the result of claw marks, was made. Such changes were hardly encountered and, if seen, not included in the investigations.

Grain Count Distributions The distributions of grain counts over labeled metaphases at the 3 successive peaks are shown in Fig 5. At the first peak (4 and 8 h) a close to normal distribution is seen with a mean grain count (MGC) of 26.5 grains/metaphase. At the second peak (32 and 36 h) a reduction in the MGC to 14.9 grains/metaphase occurred, whereas a further, slight reduction is seen at the third peak (72 and 80 h) where the MGC is 11.3 grains/nucleus. Fig 6 shows the grain distribution over labeled G_1 cells at 120 h after pulse labeling. The vast majority of labeled cells had less than 10 grains/nucleus. The results were similar for S and G_2 phase (values not shown).

DISCUSSION

The initially pulse-labeled cohort of cells is traced through the cell cycle as it leaves the S phase. This is accomplished by registration of changes in labeled cells in the various cell cycle phases with time after labeling. A peak of labeled cells thus represents

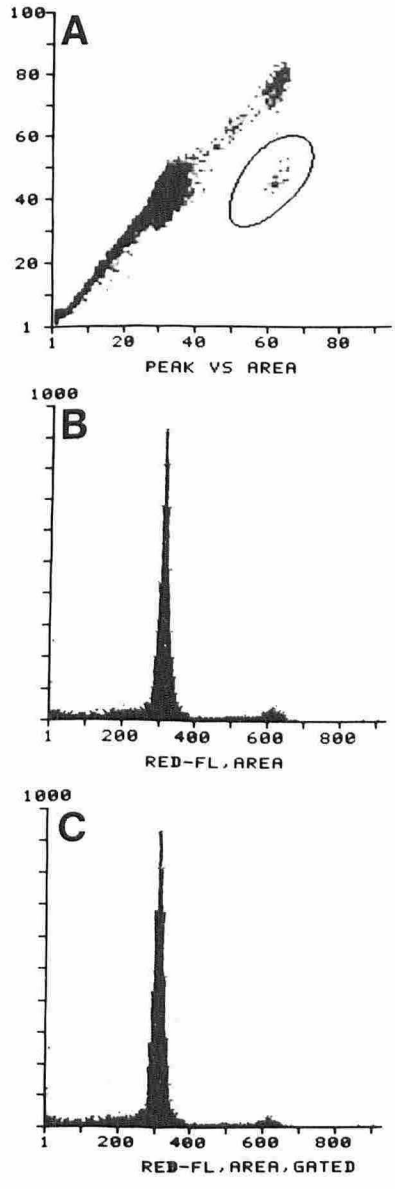

Figure 2. *A*, Two-parameter plot of DNA fluorescence from mouse epidermal basal cells. Pulse area (*abscissa*) is plotted against pulse height (*ordinate*) to discriminate G_1 doublets (*encircled dots*) from G_2 cells. G_1 doublets have the same pulse area as G_2 cells, but the pulse height is that of G_1 cells. *B*, DNA histogram of mouse epidermal basal cells where pulse area is plotted on the *abscissa* and cell number on the *ordinate*. This histogram includes G_2 doublets. *C*, DNA histogram of mouse epidermal basal cells as in (*B*) where G_1 doublets [*within the encircled area in (A)*] are omitted from the histogram by gating based on 2-parameter analysis as shown in (*A*).

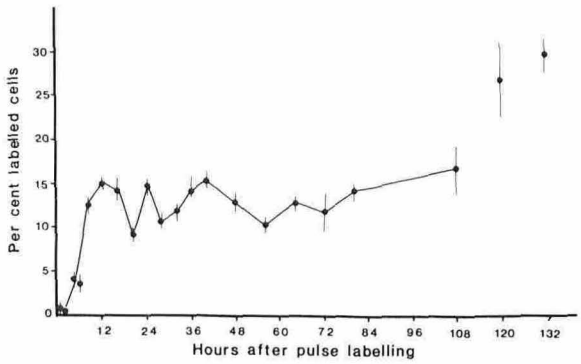

Figure 3. The proportion of labeled mouse epidermal basal cells in G_1 phase at intervals after pulse labeling (for details, see Fig 4).

the passage of the labeled cohort through that particular phase. The labeled S-phase cohort has completed one division after about 8 h, as indicated by the value of labeled G_1 cells corresponding to twice the initial labeling index at this time (Fig 3). This is consistent with corresponding phase durations [7,8], indicating that the labeling does not perturb the cell cycle traverse of labeled cells. A previous study revealed no significant changes in the circadian rhythms of cell cycle distributions after a similar [^{3}H]dThd pulse [9], both indicating that the prelabeled cohort reflects normal cell cycle kinetics.

The major observation from our studies is a significant peak of labeled mitoses at 36 h after pulse labeling, preceded and accompanied by high levels and peaks of labeled cells in S and G_2 phase (Fig 4). This shows that labeled cells have completed one cell cycle traverse in less than 30 h, and is the first report from in vivo studies of keratinocytes with such a short cell cycle time. Because the mean cell cycle time of epidermal cells from hairless mice was found to be about 55 h [7], the rapidly cycling cells must represent a subpopulation among keratinocytes that exhibits much longer cell cycle times. These cells are reflected by the high levels of labeled mitoses between 70–100 h, that also were reported by Hegazy and Fowler [10] and Potten et al [11] in mouse epidermis. Whether these cells represent one or more subpopulations with respect to cycle time, including recycling cells with short cycle time, is uncertain. The MGC of labeled mitoses in the second peak at 36 h is about half of MGC in the first peak (Fig 5), which strongly supports the theory that labeled cells have divided twice in less than 30 h. The MGC of labeled mitoses in the plateau between 70–100 h is only slightly below that in the second peak. This is consistent with labeled keratinocytes at their second division having cell cycle times in the order of 80 h, in addition to recycling of cells with short cycle times.

The data therefore show the presence of one unique and rapidly cycling keratinocyte population with cycle time less than 30 h, in addition to one or more slowly cycling populations.

Although animals with claw marks on gross examination were not used, it can be argued that skin wounds only visible upon microscopic examination might induce regenerative proliferation more easily in hairless than in haired mice. Normal growth kinetics might thus be perturbed, influencing our results. Why this is not the case is shown in the following: (a) The natural synchrony in cell division rate and estimated mean cell cycle time are similar in hairless and haired mice [12,13]. (b) The peak in percent labeled mitoses at 36 h after labeling occurs at 2000 h; at a time of the day when the circadian rhythm shows a minimum value in mitotic activity [7,9]. Since external perturbation of epidermal growth is associated with increased division activity, the rapidly cycling keratinocytes representing the peak of labeled mitoses at 36 h cannot reflect regeneration. (c) Furthermore, during epidermal regeneration in hairless mice a peak of 60% labeled S-phase cells are seen at 12 h after labeling [14], which is incompatible with the present results.

Some assumptions about a possible relationship between rapidly traversing cells and the remaining, more slowly cycling keratinocytes are to be considered. Rapidly cycling cells may have a short cell cycle time because of a temporal relationship to terminal keratinization. They may thus have stem cell properties, whereas slowly cycling cells may be more closely related to keratinization, or vice versa. A relationship between the epidermal life cycle and the length of the cell cycle, however, may not exist. Under both circumstances rapidly cycling cells may be those not halted in S or G_2 phase of the cell cycle [3]. It remains to be

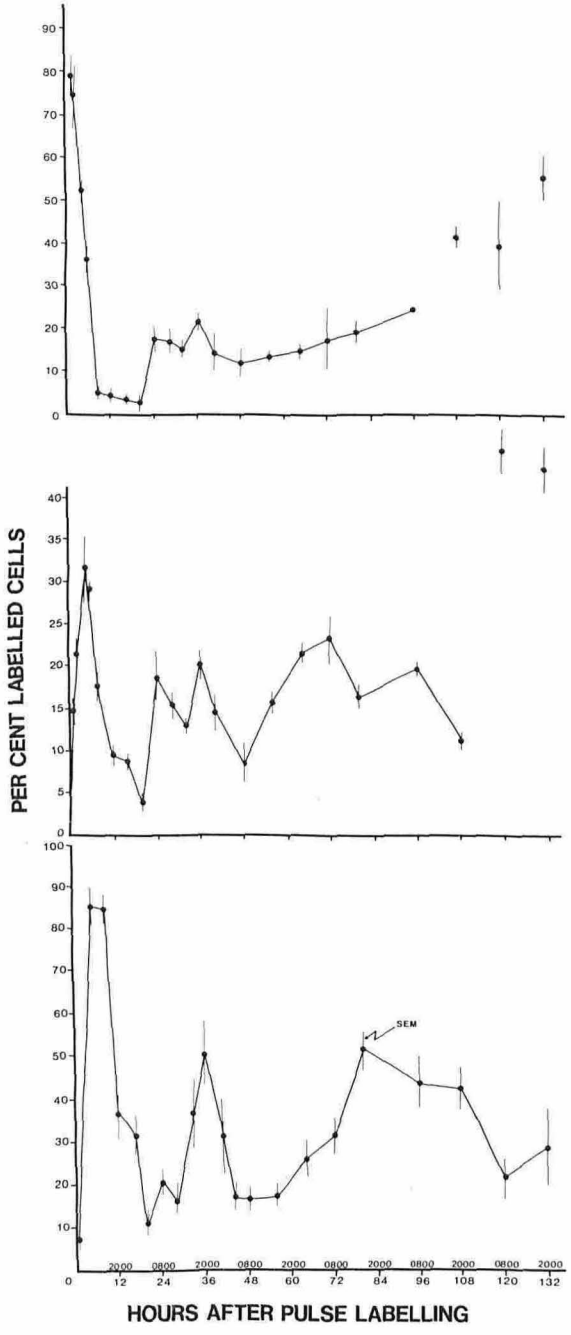

Figure 4. The proportion of labeled mouse epidermal basal cells in S phase (*upper panel*), G_2 phase (*center panel*), and metaphase (*bottom panel*) in normal mice at intervals after pulse labeling. [^{3}H]dThd (50 μCi) was injected i.p. at 0800. Cells were sorted from windows in S and G_2 phase (see Fig 1), collected on glass slides, and processed for autoradiography before counting of labeled cells. Labeled mitoses were counted in histologic sections from the same animals.

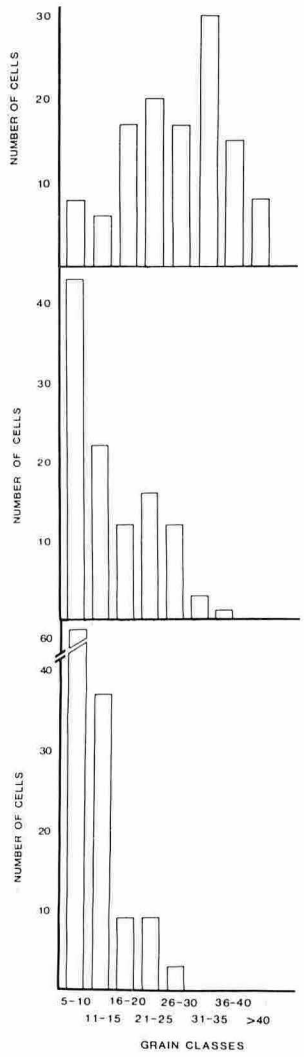

Figure 5. Grain count distributions and mean grain count (*MGC*) of labeled mitoses (from top to bottom) of the first (MGC = 26.5), second (MGC = 14.9), and third (MGC = 11.3) peaks of labeled mitoses. Each distribution reflects the pooled data from 5–10 specimens.

established whether cells are halted randomly in S and/or G_2 phase, or whether the slowly cycling cells in S and G_2 phase represent a distinct subpopulation within the epidermal life cycle.

The first peak of labeled G_2 cells reaches only 30% (Fig 4). This strongly indicates that the majority of cells in G_2 are resting or traversing very slowly through this phase compared with the labeled cohort. This is in agreement with previous findings, from which it was estimated by means of continuous [^{3}H]dThd labeling and cell sorting that about 70% of the cells in G_2 phase were delayed with a G_2 phase duration of about 32 h [3], as opposed to a mean of about 3.8 h [7]. Although slowly cycling G_2 cells contribute to a majority of the cells in G_2 phase, only a small percentage of all cycling cells are entering the slowly cycling G_2 compartment during one cell cycle [3]. We have thus confirmed by a different experimental approach the existence of slowly cycling G_2 cells. The shape and timing of the first peak of labeled mitoses which approached 100% are in accordance with previous results [1,15]. This is indicating that very few, if any, of the cells are progressing slowly through mitosis.

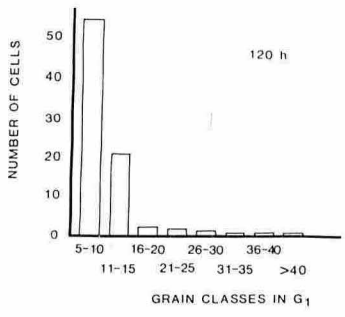

Figure 6. Grain count distribution of labeled cells in G_1 phase at 120 h after pulse labeling. Similar distributions were found also in S and G_2 phase.

The high values of labeled cells in S, G_2, and G_1 phase after 120 h are assumed partly to reflect reutilization of labeled DNA breakdown products from epidermis as well as from other tissues. This is supported by the predominance of lightly labeled cells found at these time points. This is not seen in the PLM measurements since it is necessary to keep the grain counts lower to recognize the labeled mitosis, thus not detecting the cells with small tracer activities.

Although occurring in the afternoon when the number of mitoses in hairless mouse epidermis is low [7,9,16], the second peak in the present study between 30–40 h was reproduced in a second experiment. The values from both experiments were similar and thus pooled. Detailed characteristics of epidermal subpopulations with different cell cycle times, and possible influences on the observed peaks by circadian variations, may be obtained by computer simulations of the recorded data based on different alternative models of epidermal growth organization [1,8]. Such analysis may also elucidate relationships between cells with slow and rapid S and G_2 phase traverse [2,3].

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